FERMENTATION OF RECOMBINANT *E. coli* TOP10F'/pPROEX™HTa/*BmSXP* TO ACHIEVE HIGH YIELD OF BIOMASS AND RECOMBINANT ANTIGEN FOR DIAGNOSTIC APPLICATION

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UNIVERSITI SAINS MALAYSIA

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FERMENTASI *E. coli* REKOMBINAN TOP10F'/pPROEX™HTa/*BmSXP* BAGI MENDAPATKAN HASIL BIOJISIM DAN ANTIGEN REKOMBINAN YANG TINGGI UNTUK KEGUNAAN DIAGNOSIS

oleh

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Thesis yang diserahkan untuk
memenuhi keperluan bagi
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by

KHOO TENG KEW

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LIST OF ABBREVIATIONS AND SYMBOLS

1	Alpha	α
2	Absorbance	A
3	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)	ABTS
4	Aluminium chloride hexahydrate	$AlCl_3.6H_2O$
5	Air-lift fermenter	ALF
6	Beta	β
7	*Brugia malayi*	*B. malayi*
8	Base pair	bp
9	Bovine serum albumin	BSA
10	Celsius	C
11	Calcium chloride dihydrate	$CaCl_2.2H_2O$
12	Colony forming units	CFU
13	Centimeter	cm
14	Cobalt (II) chloride hexahydrate	$CoCl_2.6H_2O$
15	Cut off value	COV
16	Central Processing Unit	CPU
17	Carbon-source	C-source
18	Copper (II) chloride dihytrate	$CuCl_2.2H_2O$
19	Column volume	CV
20	Dalton	Da
21	Digital Control Unit	DCU
22	Diurnal subperiodic	DSP
23	Dry cell weight	DCW

24	Double-distilled water	ddH_2O
25	Dissolved oxygen	DO
26	*Escherichia coli*	*E. coli*
27	For example	e.g.
28	Enzyme-linked immunosorbent assay	ELISA
29	Iron (II) sulphate	$FeSO_4$
30	Fast performance liquid chromatography	FPLC
31	Gravity	*g*
32	Gram	g
33	Global Alliance to Eliminate Lymphatic Filariasis	GAELF
34	Global Programme to Eliminate Lymphatic Filariasis	GPELF
35	Hour	h
36	Sulphuric acid	H_2SO_4
37	Boric acid	H_3BO_3
38	High cell density culture	HCDC
39	Histidine	His
40	High performance liquid chromatography	HPLC
41	Horseradish peroxidase	HRP
42	Hertz	Hz
43	Immunoglobulin E	IgG4
44	Immobilized metal affinity chromatography	IMAC
45	Institute for Research in Molecular Medicine	INFORMM
46	Isopropyl β-D-1-thiogalactopyranoside	IPTG
47	kilo Dalton	kDa
48	diPotassium hydrogen phosphate	K_2HPO_4

49	Potassium dihydrogen phosphate	KH_2PO_4
50	Kilopascal	kPa
51	Liter	L
52	Luria-Bertani	LB
53	Lymphatic filariasis	LF
54	Specific growth rate	
55	Maximum specific growth rate	$_{max}$
56	Molar	M
57	Multiple cloning sites	MCS
58	Millimolar	mM
59	Mass drug administration	MDA
60	Microfilariae	mf
61	Multi fermenter control system	MFCS
62	Microgram	g
63	Milligram	mg
64	Miligram per gram dry cell weight	$mg.g\ DCW^{-1}$
65	Magnesium sulphate heptahydrate	$MgSO_4.7H_2O$
66	Minute	min
67	Microliter	µL
68	Milliliter	mL
69	Micrometer	µm
70	Millimeter	mm
71	Manganese sulphate	$MnSO_4.H_2O$
72	Megapascal	MPa
73	Malaysian Ringgit	MYR

74	Nocturnal periodic	NP
75	Nocturnal subperiodic	NSP
76	Sodium chloride	NaCl
77	Sodium hydrogen phosphate	NaH_2PO_4
78	Sodium molybdate dihydrate	$Na_2MoO_4.2H_2O$
79	Ammonium chloride	NH_4Cl
80	Ammonium sulphate	$(NH_4)_2SO_4$
81	Nickel	Ni
82	Nickel ions	Ni^{2+}
83	Nickel- nitrilotriacetic acid	Ni-NTA
84	Nanometer	nm
85	Nitrogen-source	N-source
86	Optical density	OD
87	Open reading frame	ORF
88	Product	P
89	Percentage	%
90	Phosphate buffered saline	PBS
91	Personal computer	PC
92	Page	pg
93	Pounds per square inch	psi
94	Rotations per minute	rpm
95	Super broth	SB
96	Single-distilled water	sdH_2O
97	Sodium dodecyl sulphate polyacrylamide gel electrophoresis	SDS-PAGE

98	Stirred-tank reactor	STR
99	Terrific broth	TB
100	Tris-buffered saline	TBS
101	Tris-buffered saline Tween 20	TBS-T
102	Tricarboxylic acid	TCA
103	Total cell proteins	TCP
104	Universiti Sains Malaysia	USM
105	Volt	V
106	Volume per volume per minute	vvm
107	*Wuchereria bancrofti*	*W. bancrofti*
108	World Health Organization	WHO
109	Times	x
110	Biomass	X
111	Yield coefficient of product from substrate (Product yield)	$Y_{P/S}$
112	Yield coefficient of product from biomass (Overall specific productivity)	$Y_{P/X}$
113	Yield coefficient of biomass from substrate (Biomass yield)	$Y_{X/S}$
114	Zinc sulphate heptahydrate	$ZnSO_4.7H_2O$

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ABSTRAK

panLF Rapid™ merupakan satu ujian pantas pengesanan antibodi IgG4 berdasarkan pada pengesanan antibodi anti-filarial IgG4 yang bertindak balas dengan antigen rekombinan *B. malayi*, *BmR1* dan *BmSXP*. Kit diagnostik ini adalah sangat berguna untuk pengesanan limfatik filariasis (LF), terutamanya dalam membantu WHO dalam aktiviti sertifikasi dan pengawasan pasca-pemberian ubat secara besar-besaran selari dengan usaha Program Penghapusan LF Sedunia atau *'Global Programme to Eliminate Lymphatic Filariasis'*. Pengeluaran kit ujian ini telah menerima permintaan yang ketara di pasaran, maka peningkatan penghasilan ke skala besar dan peningkatan efisiensi penulenan adalah perlu untuk meningkatkan kadar pengeluaran dan juga mengurangkan kos pengeluaran secara besar-besaran. Dalam kajian ini hasil *BmSXP* antigen rekombinan telah dimaksimumkan melalui penghasilan biomass yang tinggi dengan menggunakan kultur sekelompok di dalam bioreaktor, dan tahap pemulihan protein sasaran ini telah dioptimumkan melalui proses penulenan hiliran. Pengkulturan bakteria rekombinan (TOP10F'/pPROEX™HTa/*BmSXP*) pada awalnya telah dioptimumkan dalam fermentasi berskala kecil dengan menggunakan kelalang goncang di mana ia menghasilkan 4.2 $g.L^{-1}$ dan 0.576 $mg.g\ DCW^{-1}$ antigen rekombinan *BmSXP*. Proses

penaikkan-skala kemudian dijalankan dengan menggunakan kaedah fermentasi kultur sekelompok di mana sel ditumbuhkan di dalam media kaldu *Terrifc broth* terubahsuai dan glukosa disuapkan secara eksponen pada kadar yang terkawal menggunakan *'Multifermenter Control Software'* (MFCS) untuk suapan secara automatik. Dengan mempelbagaikan strategi suapan kadar pertumbuhan spesifik ( ) dan strategi induksi, hasil biomass sebanyak 19.43 $g.L^{-1}$ dan 11.16 mg.g DCW^{-1} antigen rekombinan *BmSXP* telah diperolehi hasil daripada strategi suapan secara eksponen pada sebanyak 0.20 h^{-1}, dan dengan aruhan tunggal 1 mM IPTG pada akhir fasa log pertengahan pertumbuhan bakteria. Selain itu juga, dapat dilihat bahawa pada kadar suapan ini, pekali fermentasi hasil produktiviti ($Y_{P/X}$), hasil biomass ($Y_{X/S}$) dan hasil produk ($Y_{P/S}$) adalah tinggi. Strategi ini telah berjaya mengawal pengumpulan produk rencatan asid asetik di bawah tahap rencatan pertumbuhan yang dilaporkan sebanyak 2 $g.L^{-1}$ dan kestabilan plasmid didapati berada dalam keadaan baik. Antigen rekombinan *BmSXP* kemudian ditulenkan dalam keadaan tidak ternyahasli (*non-denaturing*) dengan menggunakan kromatografi afiniti tidak bergerak. Demi meningkatkan keberkesanan proses penulenan, pelbagai isipadu penimbal basuhan, kepekatan imidazol dan garam dilakukan. Didapati kepekatan garam pada 300 mM NaCl dan 30 mM imidazol memberikan hasil terbaik, dan bersama dengan 10 isipadu penimbal basuhan telah memberikan hasil antigen rekombinan *BmSXP* yang tertinggi dengan ketulenan yang baik. Tindak balas imuno dari antigen rekombinan *BmSXP* yang dihasilkan menunjukkan ia adalah 100% sensitif dan spesifik apabila diuji dengan ELISA dan Pemblotan Western menggunakan sampel serum daripada 32 pesakit LF (16 *Wuchereria bancrofti*, 16 *Brugia malayi*) dan 32 serum kawalan yang lain (16 penyakit nematoda yang lain, 16 individu sihat). Keseluruhan pengeluaran protein

sasaran dapat ditingkatkan hampir 20-kali ganda berbanding dengan kaedah pengkulturan konvensional di dalam kelalang. Kesimpulannya, kajian ini telah memberikan kaedah yang lebih baik, lebih efisien dan menjimatkan kos untuk proses pengeluaran dan penulenan protein rekombinan *BmSXP*.

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ABSTRACT

panLF Rapid™ is a rapid IgG4 antibody detection test which is based on the detection of anti-filarial IgG4 antibodies that react with recombinant *B. malayi* antigens, *BmR1* and *BmSXP*. This diagnostic kit is very useful for the detection of lymphatic filariasis (LF), especially in assisting the WHO on its certification and surveillance activities of post-mass drug administration that is in relation to its 'Global Programme to Eliminate Lymphatic Filariasis' effort. The production of this test kit has received a significant demand in the market, hence there is a need to up-scale the production of the recombinant antigens and increase the purification efficiency in order to increase the production rate and also reduce the cost of production. In this study the yield of *BmSXP* recombinant antigen was maximized by achieving high biomass yield using fed-batch culture in a bioreactor, and the recovery rate of the protein of interest was optimized in the downstream purification process. The cultivation of the recombinant bacteria (TOP10F'/pPROEX™HTa/*BmSXP*) was initially optimized in small-scale fermentation using shake flask culture where it yielded 4.2 $g.L^{-1}$ and 0.576 $mg.g\ DCW^{-1}$ of *BmSXP*. The up-scaling process was then performed using fed-batch fermentation where cells were grown in modified Terrific broth medium and

glucose was fed exponentially at a controlled rate using Multifermenter Control Software (MFCS) for automated feeding. Varying an assortment of feeding strategies, specific growth rate (μ) and induction strategies, biomass concentration of 19.43 $g.L^{-1}$ and 11.16 $mg.g\ DCW^{-1}$ of *BmSXP* were obtained based on exponential feeding strategy at μ of 0.20 h^{-1}, and with 1 mM single pulse IPTG induction at the late-log phase of the bacterial growth curve. It was also observed that at this feeding rate, the fermentation yield coefficients of overall specific productivity ($Y_{P/X}$), biomass yield ($Y_{X/S}$) and product yield ($Y_{P/S}$) were high. This strategy has successfully controlled the accumulation of acetic acid by-inhibitory product below the reported growth inhibitory level of 2 $g.L^{-1}$ and plasmid stability was found to be good. The *BmSXP* recombinant antigen was then purified under non-denaturing conditions using immobilized metal affinity chromatography. In order to increase the efficiency of the purification process, various volumes of wash buffer, imidazole and salt concentrations were performed. Salt at 300 mM and imidazole at 30 mM were found to be the best concentrations, and along with 10 column volumes of washing buffers gave the best yield of *BmSXP* recombinant antigen while achieving sufficient purity. Immunoreactivity of the recovered *BmSXP* recombinant antigen was found to be 100% sensitive and specific when tested with ELISA and Western blot using serum samples from 32 LF patients (16 *Wuchereria bancrofti*, 16 *Brugia malayi*) and 32 other control sera (16 other nematode disease, 16 healthy individuals). The overall production of the target protein was improved to almost 20-fold compared to the conventional flask cultivation method. In conclusion, this study provided an improved, more efficient and cost-saving method for the production and downstream processing of *BmSXP* recombinant protein.

CHAPTER I

INTRODUCTION

1.1 Introduction to filariasis

1.1.1 Lymphatic filariasis

Lymphatic filariasis (LF) or elephantiasis as commonly known, is a parasitic disease caused by thread-like filarial nematodes or round worms that live in the human lymphatic system. LF is mainly caused by three species of filarial nematodes namely *Wuchereria bancrofti* (*W. bancrofti*)*, Brugia malayi* (*B. malayi*) and *Brugia timori*.

This disease is widespread throughout countries located within the equator band, namely the tropical and sub-tropical regions of the world, such as Asia, Africa, Central and South America. An estimated 1.3 billion people around the world (approximately 19% of world population) are at risk of LF infection. Southern and Southeast Asian regions have by far the greatest number of people (891 million) at risk for LF (accounting for 68% globally), out of which 454 million people at risk are in India alone. Tropical Africa represents the second largest number of people at risk, estimated at 382 million in 2007 (30% globally). Currently over 120 million people in at least 83 countries are already infected, with more than 51 million in chronic stage whereby they have been incapacitated or disfigured with swollen breasts (lymphoedema) and genitals (hydrocele) or swollen limbs with thickened, hard, rough and fissured skin, a condition known as Elephantiasis (Michael & Bundy, 1997; Lindsay & Thomas, 2000; Muturi *et al.*, 2008; WHO, 2008; GAELF, 2010). In addition to the overt abnormalities, internal damage to the kidneys and lymphatic system is a common and hidden problem (Srivastavaa *et al.*, 2010). The economic

impact of LF is significant as it is one of the world's most disabling and disfiguring diseases. This disease strikes poverty ridden and underdeveloped countries, hence it is also known as the disease of poverty.

In 1998, the World Health Organization (WHO) has identified lymphatic filariasis to be one of the six infectious diseases that has the potential to be eliminated as a public health problem (WHO, 1998; Ottesen *et al.*, 2008). In response to this, Global Programme to Eliminate Lymphatic Filariasis (GPELF) was initiated in year 2000 with two major objectives to achieve. Firstly is to interrupt transmission of the parasite and the other objective is to provide care for those who suffer the devastating clinical manifestations of the disease (morbidity control). The ultimate ambitious goal of this program is to relegate LF from the world as non-public health priorities by year 2020 (Addis & Brady, 2007).

1.1.2 *Wuchereria bancrofti*

Bancroftian filariasis is caused by *W. bancrofti* infection and it is responsible for 90% (115 million) of all LF infections. *W. bancrofti* largely affects areas across the broad equatorial belt (Africa, the Nile Delta, Turkey, India, the East Indies, Southeast Asia, Philippines, Oceanic Islands, Australia, and parts of South America). The remaining 10% are due to two species of the genus *Brugia* and occur typically in Asia (Figure 1.1) (Michael & Bundy, 1997; Fischer *et al.*, 2004; WHO, 2006).

The adult *W. bancrofti* male and female worms' measure 0.2 mm wide and up to 10 cm long (Figure 1.2). Mature male and female worms mate in the lymphatic system of the definitive host, and females could produce copious numbers of up to 50,000 microscopic microfilaria (mf) per day, each mf measures 250–300 mm long,

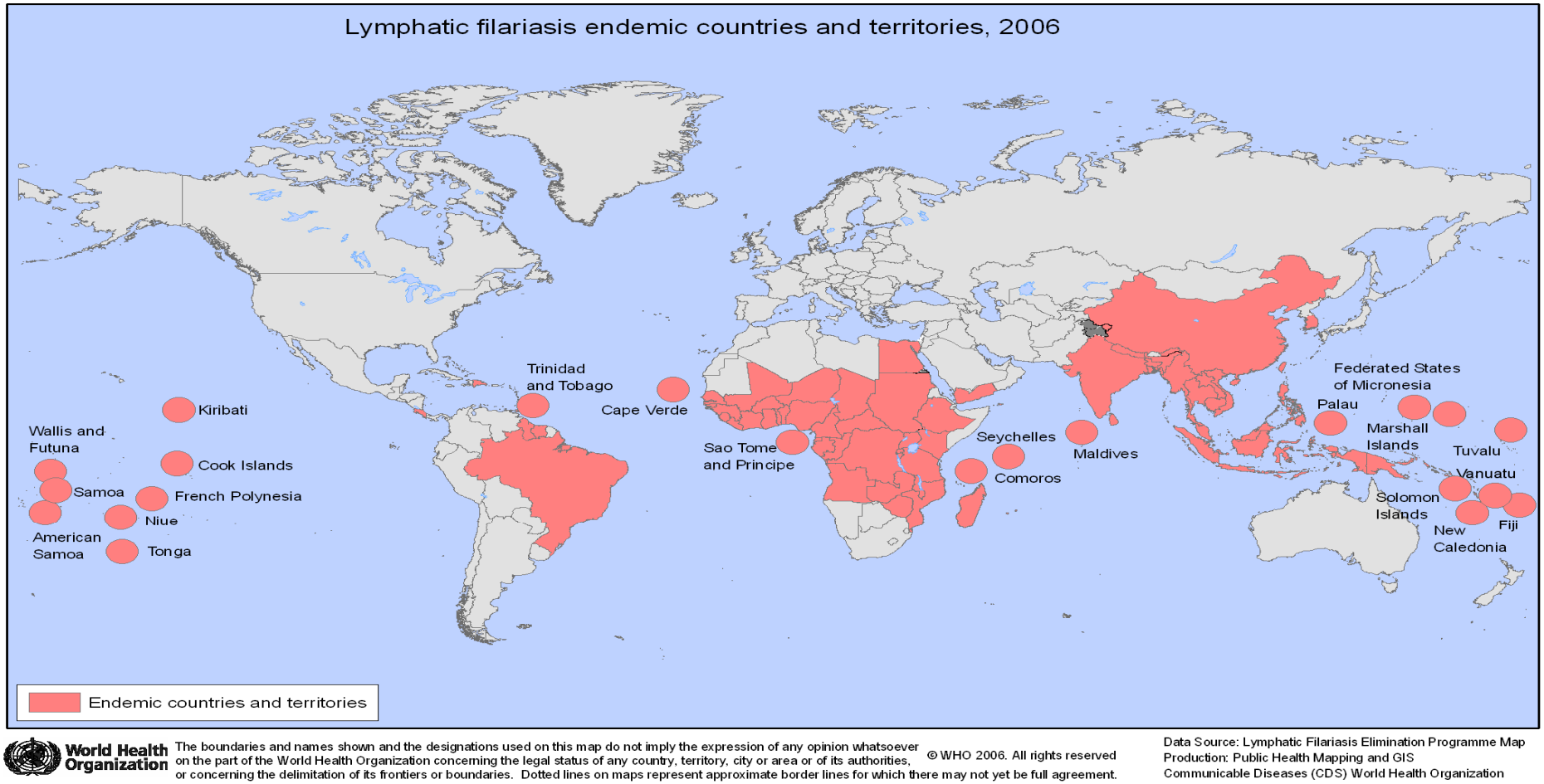

Figure 1.1 Lymphatic filariasis endemic areas

Source: WHO (2008) and Manguin *et al.* (2010)

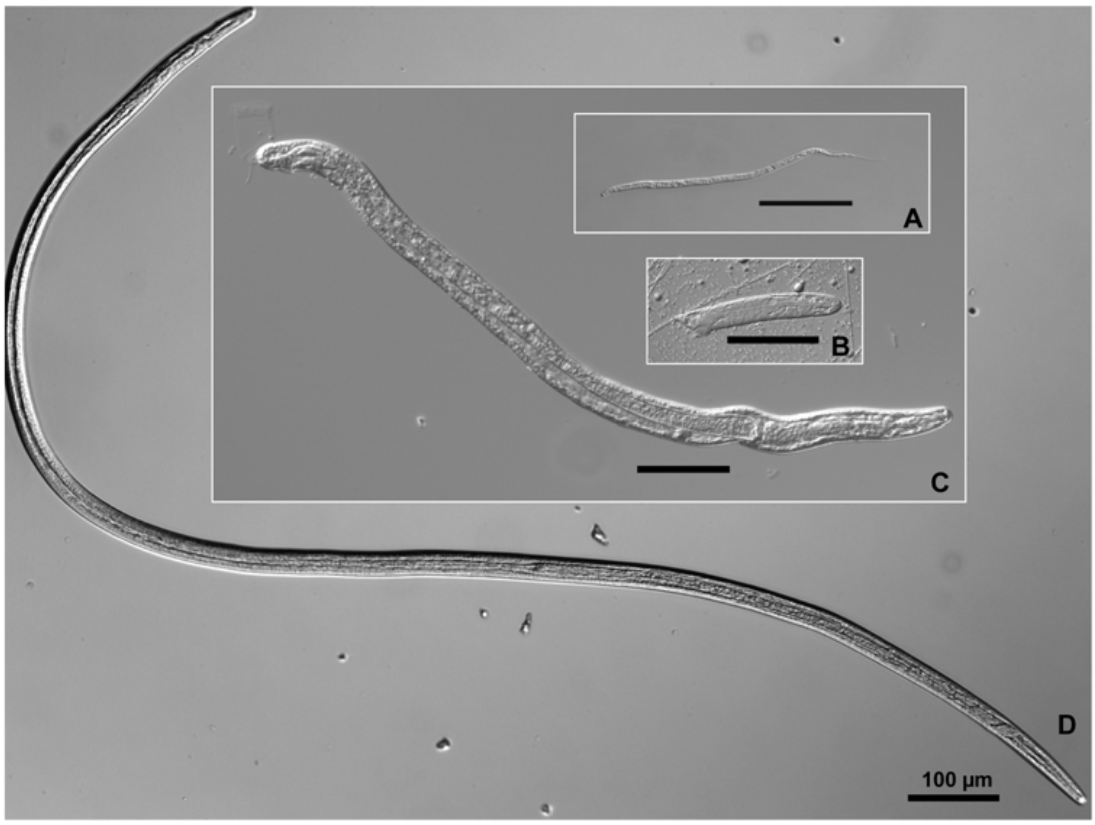

Figure 1.2 Relative sizes of mf developmental stages that occur within compatible mosquito hosts: Cyclodevelopmental transmission

Source: Erickson *et al. (2009)*

A: Microfilariae are ingested during blood feeding

B: Parasites differentiate into non-feeding, first-stage larvae within mosquito indirect flight muscle cells

C: Following the first molt, second-stage larvae remain intracellular parasites which ingest cellular material into their newly developed digestive tract

D: Third-stage larvae leave the muscle cells and migrate to the mosquito's head and proboscis where they will exit through the mosquito cuticle during blood feeding

8 m wide. These microscopic mf would then find their way into the blood circulation, survive and circulate freely in the blood of the human host for many months, possibly longer, while awaiting an opportunity of being picked up by mosquitoes and continue their life cycle (Section 1.1.3). Adult worms live for an estimated four to six years, but may survive up to 15 years or more with each producing ten million of mf in their lifetime (Manguin *et al.*, 2010).

1.1.3 Transmission and life cycle

There are five different stages in the life cycle of lymphatic filaria as depicted in Figure 1.3. Filariasis is spread from an infected human whom someone with worms in his/her bloodstream to an uninfected human by mosquitoes. More than 70 species and subspecies of mosquitoes mainly *Anopheles, Aedes*, *Culex* and *Mansonia* can transmit the infection (Stone *et al*., 1959; Nanduri & Kazura, 1989).

During the blood meal, mosquito vectors ingest mf produced by adult female worms found circulating in the peripheral blood. Within 2 hours time, the mf will quickly penetrate the midgut epithelium to access the hemocoel (Christensen & Sutherland, 1984). Mf then migrate in the mosquito's hemolymph to reach the thoracic musculature and from there penetrate into the indirect flight muscles. It is in the thoracic muscle tissue where larvae development take place, where mf undergo two molts (L_1 and L_2). After several days, the parasites undergo an additional molt and emerge as infective-stage larvae (L_3). Approximately 10 to 14 days after exposure depending to environmental temperature, the L_3 eventually breaks free from the flight muscles into the hemocoele and ultimately migrate to the insect's head lodged in or near the labium of the proboscis. When the mosquito returns to blood feed, these 1.2–1.6 mm long L_3 infective larvae will break through the cuticle or emerge

from the tip (labellum) of the mosquito's labium onto the vertebrate human host skin. The parasite is thus indirectly transmitted and must enter the host body via an open portal, such as the mosquito bite wound or a nearby break in the skin.

After entering the definitive host body, the L_3 is then transported via the lymphatic vessels to lymph nodes to begin development (following two intermediate molts) into mature adult male or female worms (0.2 mm wide and up to 10 cm long) where they mate and the females produce copious numbers of up to 50,000 microscopic mf per day (250–300 mm long, 8 mm wide). For *W. bancrofti* it takes a period of four to 15 months (possibly longer) before the appearance of mf in the peripheral blood. Adult worms live for an estimated four to six years, but may survive up to 15 years or more with each producing ten of millions of mf in their lifetime. Mf are believed to survive and circulate freely in the blood of the human host for many months, while awaiting an opportunity of being picked up by mosquitoes (Erickson *et al.*, 2009).

1.1.4 Clinical manifestation

Although infection from bancroftian filariasis is not fatal, it is considered a leading cause of infirmity, permanent disability and chronic morbidity, often resulting in societal stigma of disfigured victims (Figure 1.4).

There are two types of clinical manifestation, namely: lymphatic filariasis (presence of adult worms) and occult filariasis (immuno hyper responsiveness). In LF clinical manisfestation, it could further sub-categorized into 4 stages, namely: asymptomatic amicrofilaraemia stage, asymptomatic microfilaraemia stage, stage of acute manifestation and stage of obstructive (chronic) lesions.

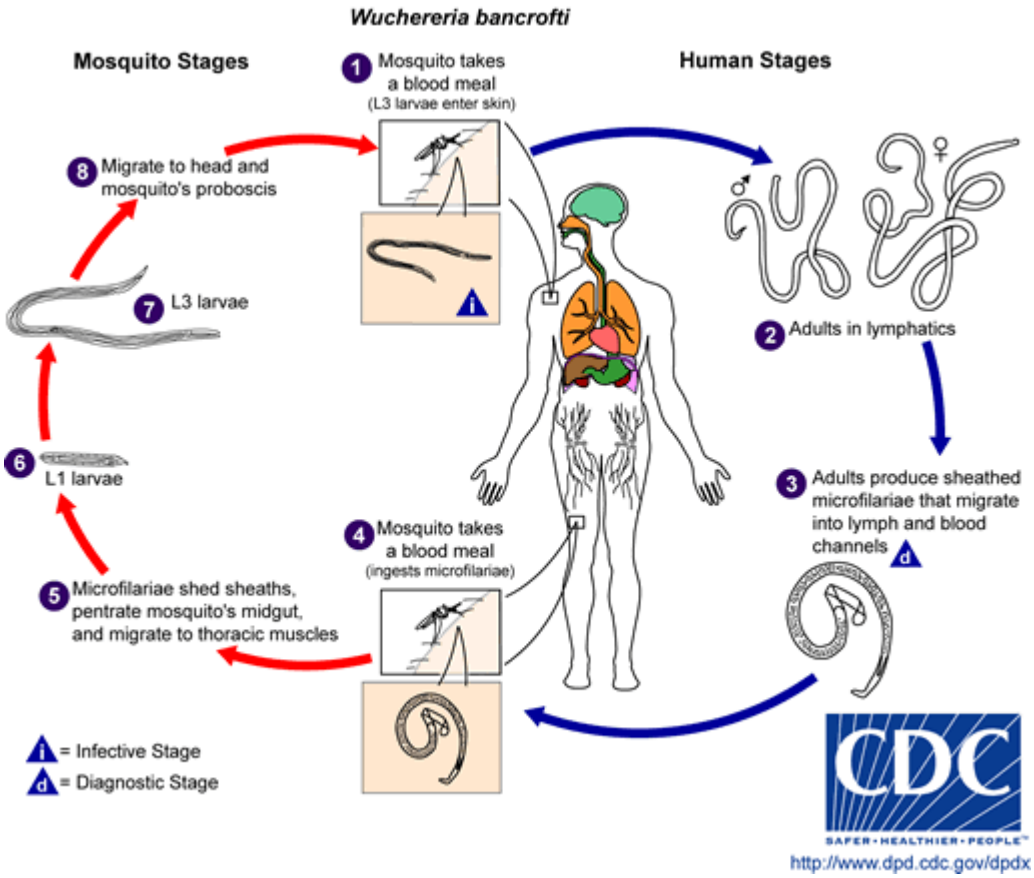

Figure 1.3 The life cycle of *W. bancrofti*

Source: Life Cycle of *Wuchereria bancrofti* (2010)

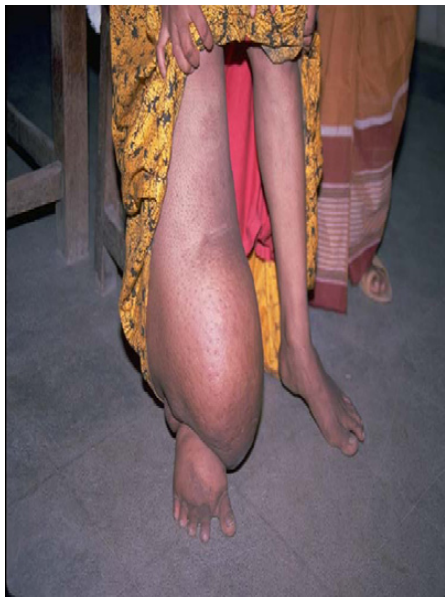

Figure 1.4 Elephantiasis (lymphoedema) of lower limb

Source: Wuchereria bancrofti (2010)

Asymptomatic microfilaraemia individuals are the infected people who do not exhibit any physicals symptom for months and years, even though they have circulating mf making them an important source of infection. Individual with acute symptoms may experience fever, chill, malaise, headache and vomiting. It may take 10-15 years to reach the chronic (obstructive) lesions stage whereby lymphoedema occurs, resulting in temporary or permanent infirmity. This is due to the permanent damage to the lymph vessels caused by the adult worms. Elephantiasis, is the result of lymphoedema of the extremities, and frequently associated with lymphadenopathy, lymphangitis, hydrocoele (in males) and chyluria (Crompton & Savioli, 2007). This often led to painful and gross enlargement of the legs (Figure 1.4) and arms, the genitals, vulva and mammary glands.

Occult or cryptic filariasis is a classical clinical manifestation in which mf will not be present. Occult filariasis is believed to be the result of hyper responsiveness to filarial antigens derived from mf (seen more in males). Patients present symptoms with paroxysmal cough and wheezing, low grade fever, scandy sputum with occasional haemoptysis, adenopathy and increased eosinophilia (Ganesh, 2010).

1.1.5 Diagnosis of lymphatic filariasis

Diagnostic tools are important to GPELF because they affect decisions on where to distribute mass drug administration (MDA), how to measure its effectiveness, how to define targets and endpoints for stopping MDA and how to monitor populations for resurgence of LF transmission following suspension of MDA (WHO, 2005).

The standard method for diagnosing active infection is the identification of mf in a blood smear by microscopic examination. There are three variants of *W. bancrofti*

recognized on periodicity patterns of circulating mf found in peripheral blood of humans, namely: the nocturnal periodic (NP), nocturnal subperiodic (NSP) and diurnal subperiodic (DSP) forms. Periodicity is based on the prevailing circadian distribution of mf in the peripheral blood. NP form presents the majority of mf by night (peak periodicity at 2200 hours to 0300 hours) with very few observable by day as they sequester in the lungs. The two subperiodic forms (NSP and DSP) are far more restricted in distribution (Figure 1.1) and are present in the peripheral blood 24 hours a day with peak densities typically seen in the late afternoon and early evening hours (1800 hours to 2000 hours) (Gould *et al.*, 1982). Since most prevalent LF endemic areas consist of mf in NP variant, blood collection should be done at night to coincide with the appearance of the mf. A thick blood smear is made by spreading a drop of blood onto the slide, dried and stained with Giemsa or hematoxylin and eosin, before examining the prepared slide under the microscope for the presence of mf. The advantages of thick blood smear technique are specificity, inexpensive and requires little infrastructure. However, this method is insensitive for active infections as it misses people with low mf counts and those with amicrofilaremic infections who are individuals that have the potential to contribute to future transmission. In addition, night blood collection do not have the desirable features in practice because proper sampling of populations, preparation of smears, staining and microscopy are labor intensive is troublesome to both the staff and villagers and impractical in some endemic areas (Weil & Ramzy, 2007). For increased sensitivity, concentration techniques can be used.

In the not-so-distant past, diagnostic tools for LF were limited to clinical examination and the detection of mf. However, with the recent advances in filarial diagnostics over the years, molecular diagnostic tools such as sensitive PCR assays have been

developed to detect DNA of lymphatic filarial parasites in humans and in mosquito vectors (Fisher *et al.*, 2002). Although rapid methods for the detection of PCR products have been established (Fisher *et al.*, 2002 & Klüber *et al.*, 2001), the main obstacles preventing its practice is that PCR assays require a sophisticated laboratory infrastructure and trained skilled personnel to perform the analysis. PCR assays also require long running time up to several hours prior to data collection, thus it is not practical to be used with large number of samples and for field screening. In addition, PCR generally do not detect people with amicrofilaraemic infection.

Another detection method is the usage of ultrasonography whereby it uses a 7.5 MHz or 10 MHz probe to locate and visualize the movements of living adult worms of *W. bancrofti* in the lymphatic vessels of asymptomatic males with microfilaraemia, also known as the search for the 'filarial dance sign'. However, this technique is not suitable for large scale studies, and it is not very useful for brugian filariasis diagnosis in which the adult worms are not found in the peripheral lymphatics.

Lymphoscintigraphy is another known technique used to diagnose LF. The structure and function of the lymphatics of the involved limbs are assessed by lymphoscintigraphy through the injection of radio-labelled albumin or dextran in the web space of the toes. The structural changes are then imaged using a Gamma camera. Lymphatic dilation and obstruction can be directly demonstrated even in early clinically asymptomatic stage of the disease. However, the disadvantage of this technique is again the requirement of a sophisticated laboratory infrastructure and trained skilled personnel to perform the analysis.

Last but not least, immunoassays detect the presence of specific antigens or antibodies in the blood of individuals. Rapid tests kits such as antibody-based

detection test kits are now commercially available to detect brugian filariasis (Rahmah *et al.*, 2003), bancroftian filariasis (Weil *et al.*, 1997) and both kinds of filariasis (Rahmah *et al.*, 2007). To-date, this technique has shown tremendous potential in field application as the preferred diagnostic method of filarial infection attributed by its features of not requiring blood sampling at certain time of the day, high sensitivity and specificity, rapid (approximately 15 minutes to reading), and user friendliness. Immunoassays have performed their function well as the diagnostic tools used to assist and facilitate surveillance activities in monitoring the control efforts, and to evaluate new drugs.

1.1.6 Elimination of filariasis

The World Health Organization (WHO) has identified LF to be one of the six infectious diseases that has the potential to be eliminated as a public health problem (WHO, 1998; Ottesen *et al.*, 2008). This would be done using selective diagnosis to identify endemic areas followed by repeated cycles of MDA to reduce both infection prevalence and transmission rates to levels below those required for sustained transmission (Ottesen *et al.*, 1997; Molyneux, 2001; Ottesen, 2006). In this case, a single dose of two drugs regimens has being advocated (albendazole 400 mg plus diethylcarbamazine (DEC) 6 $mg.kg^{-1}$, or albendazole 400 $mg.kg^{-1}$ plus ivermectin 200 $\mu g.kg^{-1}$ for a period of 4-6 years corresponding to the reproductive life span of the parasite (Ottesen, 2000).

In response to this, the Global Alliance to Eliminate Lymphatic Filariasis (GAELF) was formed in the year 2000, with the sole purpose of supporting GPELF based on MDA. This programme aims to eliminate LF by interrupting the transmission of infection and to alleviate and prevent both suffering as well as disability caused by

the disease. The principal strategy for the latter focuses on decreasing the secondary bacterial and fungal infection of limbs and genitals (Ottesen, 2000). The ultimate ambitious goal of this program is to relegate LF from the world as non-public health priorities by year 2020 (Addis & Brady, 2007). With a target population of 1.3 billion people, GPELF is the largest infectious disease intervention based on MDA initiated to date (WHO, 2008).

Mapping and surveillance studies play a determining role in the success of GPELF. LF endemic areas need to be identified in order to allow repeated cycles of MDA to be carried out to reduce both infection prevalence and transmission rates to levels below those required for sustained transmission (Ottesen *et al.*, 1997; Molyneux, 2001; Ottesen, 2006). Hence, sensitive and specific diagnostic tools are required to assist and facilitate mapping and surveillance activities in monitoring the control efforts of the programme.

1.1.7 panLF Rapid™

Since most of the endemic areas (Figure 1.1) reside in areas which are remote and/or without adequate health and laboratory facilities, therefore a rapid and field applicable diagnostic test, particularly those based on immunochromatography technology, are most suitable to be employed for the GPELF to ensure that it can be performed easily by field workers while also giving reliable and reproducible results.

Immunochromatography have became the most practical field applicable solution due to its attributes of easy on-site testing, followed by rapid, simple reading and interpretation of results. These features would avoid potential logistical challenges for sample storage and transportation, as well as more serious problems such as

sample mix-up due to unclear/unreadable labels and sample degradation that may occur if collection and performance of tests are not conducted at the same or nearby locations (Rahmah *et al.,* 2007).

panLF Rapid™ (Figure 1.5) is a rapid immunochromatographic test strip that utilizes *BmSXP* and *BmR1* recombinant antigens for the detection of specific IgG4 antibodies against LF parasites of both bancroftian and brugian filariasis (Rahmah *et al.,* 2007). The test strip consists of three lines namely two test lines, one comprising *BmSXP* and the other *BmR1* recombinant antigens, and a final control line. Goat anti-mouse IgG antibody is employed in the control line. Serum/plasma and whole blood are employed as test samples. These test lines are invisible in an unused test and are coloured red after performance of the test due to the reaction between the anti-filarial antibodies in patient sera with the colloidal gold conjugated monoclonal anti-human IgG4. The test is performed by delivering 25 µl serum sample into the square bottom well of the test strip. When the sample front reaches the blue line on the cassette window, two drops of buffer are then added to the top oval well to release the conjugate solution (monoclonal anti-human IgG4 conjugated to colloidal gold). This is followed by pulling a plastic tab at the bottom of the cassette and adding a drop of buffer into the square bottom well. The results are then ready to be read 15 minutes later. If only one red band appeared at the control line, this denotes a negative result. A test is interpreted as positive when either three red lines (two test lines and a control line) or two red lines (a test and a control line) are observed (Rahmah *et al.,* 2007; MBDr, 2010).

BmSXP is a recombinant antigen derived from *SXP1* gene [GenBank no: M98813]. The clone was isolated from a *B. malayi* adult male worm cDNA library with sera of

Figure 1.5 panLF Rapid™ rapid immunochromatographic diagnostic kit for the detection of both bancroftian and brugian filariasis infection

bancroftian filariasis patients (Dissanayake *et al.,* 1992). A rapid flow-through IgG immunofiltration test using *Wb*SXP recombinant antigen has been developed and a sensitivity of 91% (30/33) was recorded for detection of *W. bancrofti* infection (Lammie *et al.,* 2004). The other recombinant antigen, *BmR1* which was derived from *Bm17DIII* gene [GenBank: AF225296] has shown to be highly sensitive (>95%) and specific (≥ 99%) for the detection of *B. malayi* and *B. timori* infections in laboratory evaluations (Rahmah *et al.,* 2003; Lammie *et al.,* 2004; Fischer *et al.,* 2005) and field studies (Supali *et al.,* 2004; Jamail *et al.,* 2005; Melrose & Rahmah, 2006). *BmSXP* was found to be more sensitive (95%) in detecting *W. bancrofti* infection as compared to *BmR1* (14%). On the other hand *BmR1* was more sensitive than *BmSXP* in detecting *B. malayi* infection (98% and 84% respectively) (Rohana *et al.,* 2007). Since *BmR1* and *BmSXP* recombinant antigen cross-reacts with bancroftian and brugian filaria infection sera respectively, the panLF Rapid™ test is not useful for species identification. However in the context of GPELF, this does not pose a problem. When both recombinant antigens were applied to the panLF Rapid™ test strip, a multicenter evaluation conducted in 2007, has shown an average overall sensitivity of 96.5% (390/404); with the sensitivity for the detection of *W. bancrofti* infection at 96.0% (217/226), while the detection of brugian filariasis was 97.2% (173/178). Average specificities of 99.6% were recorded when evaluated with serum samples from a large variety of other infections, which included helminthes, protozoan, bacterial and viral infections (Rahmah *et al.,* 2007).

In the pre-certification phase of the elimination program and in the surveillance activities post-elimination, a highly sensitive test as displayed by an antibody-based diagnostic tool is essential since the level of infection (if any) is very low. Therefore, although a rapid antigen detection test is already available for bancroftian filariasis,

an antibody detection assay would probably be more useful in the screening of young children as required in the precertification phase of GPELF. Antigen detection assays depend on the presence of developmentally mature worms while antibody assays could potentially detect exposure to infective larvae by children.

A rapid test such as panLF rapid™ would be very useful in several kinds of situations, namely testing in areas where there are mixed bancroftian and brugian filaria infections, in areas where the infecting species is not known or not confirmed, and for screening of immigrant workers in countries such as Malaysia which has more than 1.3 million workers from filarial endemic countries. These workers may pose a threat to the achievement of the disease elimination or they may be a source of resurgence of the disease in the future (Rahmah *et al.,* 2007).

1.2 *E. coli* fermentation

1.2.1 Introduction

Industrial microbiology is where microorganisms are put to work in order to yield a product. Fermentation is an important part of industrial microbiology, and it is defined as the process of deriving energy from the oxidation of organic compounds via an electron transport chain (Klein *et al.*, 2004). In general, fermentation involves the breaking down of complex organic substances into simpler ones. The microbial or animal cell obtains energy through glycolysis, splitting a sugar molecule and removing electrons from the molecule. The electrons are then passed to an organic molecule such as pyruvic acid. This results in the formation of a waste product that is excreted from the cell, such as ethyl alcohol, butyl alcohol, lactic acid, and acetone. Nevertheless, the saying goes: “One man’s trash is another man’s gold”. The unwanted waste product from the cultured cells is then harvested and purified into

valuable industrial products which would then be sold to the supply chain. To-date, it is now the primary method of bioproduction in the biotechnology industry.

Escherichia coli (*E. coli*) has been the preferred "workhorse" for the production of recombinant proteins as it is the best characterized prokaryotic host in terms of molecular genetics, physiology, and expression system (Makrides, 1996; Choi & Lee, 2004; Choi *et al.,* 2006). These attributes contributed in it being selected as one of the pioneer organisms chosen for large scale fermentation studies.

Exploring the growth limits of microorganisms in general and *E. coli* in particular, engaged industrial microbiologists many years before it was possible to convert *E. coli* to a "production machine" for heterologous proteins. Early studies on high cell density growth of *E. coli* were performed either to investigate the limits of bacterial growth in liquid cultures (Gerhardt & Gallup, 1963) or to obtain large quantities of exponentially grown *E. coli* needed for biochemical studies (Bauer & Ziv, 1976). During the 1980s, when much information on the genetics and physiology of the bacterium accumulated and *E. coli* became the obvious organism of choice for recombinant protein production (Lee, 1996), much more emphasis was put on its high-density growth. Since then, numerous methods to obtain high-density cultures have been developed, each aiming at providing means to bypass the physiological constrains that prevent bacteria from growing to the limit of physical barriers between solid state and liquid suspension of cells (Shiloach & Fass, 2005).

Large scale fermentation in bioreactors of all sizes has been employed for the purpose of increasing volumetric productivity of the cultured *E. coli* by achieving high cell density culture. In addition, other advantages of bioreactor fermentation technology are increased cost-effectiveness, reduced culture volume, enhanced

downstream processing, reduced wastewater, lower production cost and a reduced investment in equipment (Choi *et al.,* 2006). The development of this technique for *E. coli* has facilitated the production of recombinant proteins and non-protein biomolecular products such as amino acids, primary and secondary metabolites with high productivities (Jeong & Lee, 1999; Gerigk *et al.,* 2002; Choi *et al*., 2006).

1.2.2 Recombinant protein production in *E. coli*

Escherichia coli (*E. coli*) is a Gram negative, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms (endotherms). *E. coli* has been the most frequently employed host due to the vast availability of numerous expression systems designed for producing recombinant proteins (Makrides, 1996). In addition, the bacteria can also be grown easily and its genetics are comparatively simple and easily manipulated or duplicated through a process of metagenics, making it one of the best-studied prokaryotic model organisms for biotechnology and microbiology. The many advantages of *E. coli* have ensured that it remains a valuable organism for the high-level production of recombinant proteins (Gold, 1990; Hodgson, 1993; Olins & Lee, 1993; Shatzman, 1995; Georgiou & Valax, 1996).

However, in spite of the extensive knowledge on the genetics and molecular biology of *E. coli*, not every gene can be expressed efficiently in this organism. This may be due to the unique and subtle structural features of the gene sequence, the stability and translational efficiency of mRNA, the ease of protein folding, degradation of the protein by host cell proteases, major differences in codon usage between the foreign gene and native *E. coli*, and the potential toxicity of the protein to the host. Moreover, the major drawbacks of *E. coli* as an expression system include the

inability to perform many of the post-translational modifications found in eukaryotic proteins, the lack of a secretion mechanism for the efficient release of protein into the culture medium, and the limited ability to facilitate extensive disulfide bond formation (Makrides, 1996). In addition, the stability of foreign proteins produced in *E. coli* can be low due to proteolytic degradation, and overexpressed proteins are often produced in the form of inclusion bodies, which later require complicated and costly denaturation and refolding processes to make them functional.

A variety of techniques including the use of different promoters and host strains, coexpression of chaperones and changing cultivation conditions have been employed to solve some of these problems. In addition, researchers have developed various methods to direct recombinant proteins to different cellular compartments (Makrides, 1996; Choi & Lee, 2004). Figure 1.6 summarizes various strategies employed for the production of recombinant proteins in *E. coli*.

E. coli cells consist of inner and outer membranes that divide the organism into three compartments: the cytoplasm, the periplasm and the extracellular space, out of which the recombinant proteins can be targeted to one of these compartments. The choice of an expression system for high-level production of recombinant proteins depends on many factors such as cell growth characteristics, expression levels, intracellular and extracellular production, and the biological activity of the target protein. In addition, each expression system has specific costs in terms of process, design, and other economic considerations (Choi *et al.,* 2006). The decision to target recombinant proteins to the cytoplasmic space, periplasmic space or culture medium depends on balancing the advantages and disadvantages of each compartment as tabulated in Table 1.1.

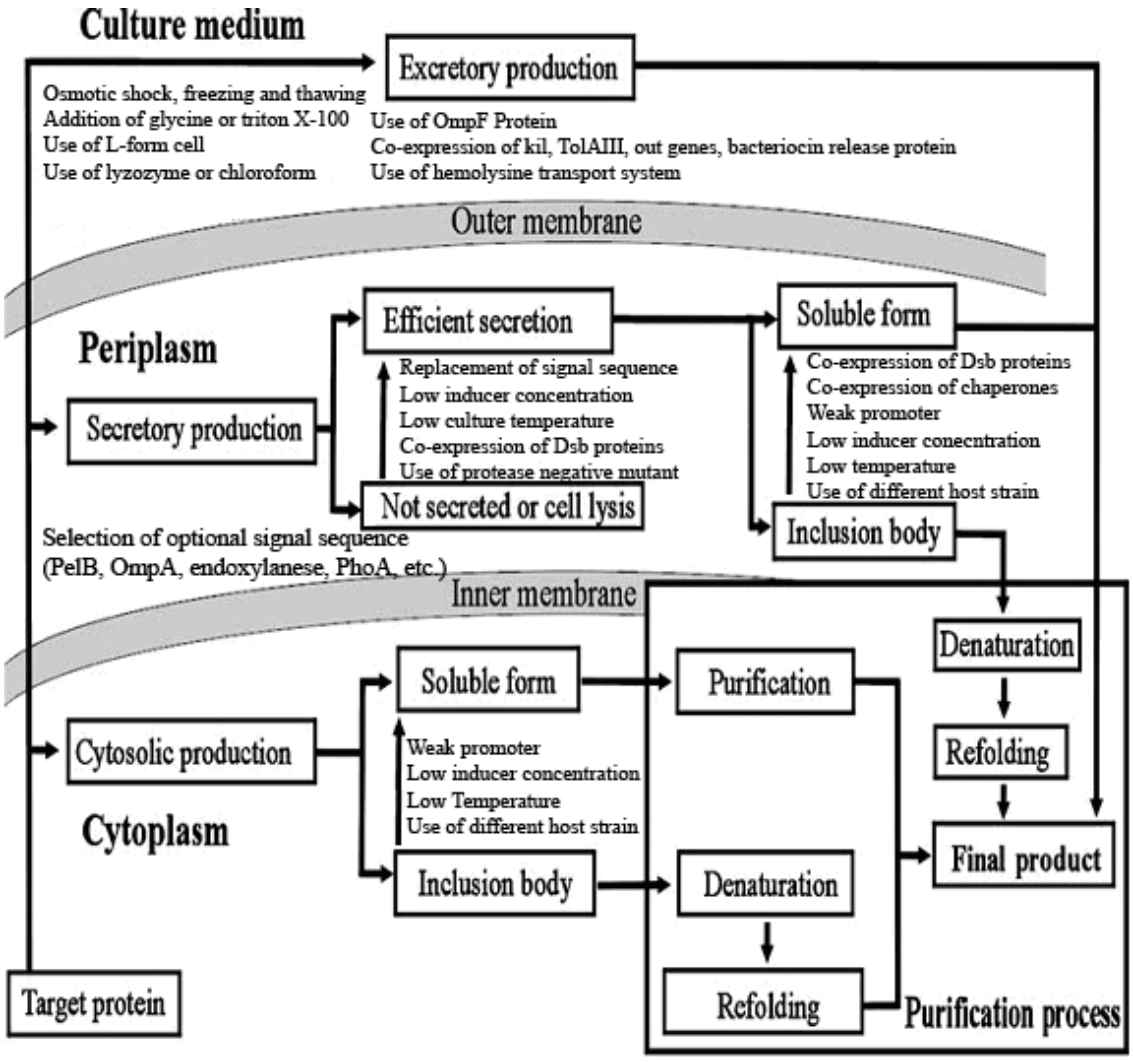

Figure 1.6 Strategies for the production of recombinant proteins in *E. coli*

Source: Choi *et al.* (2006)

Table 1.1 Advantages and disadvantages of protein production at different compartments of *E. coli*

	Advantages	Disadvantages
Cytosolic production	- Higher protein yield. - Simple plasmid construct. - Inclusion body, thus easy purification, protection from proteases, and inactive protein (non-toxic).	- N-terminal extension. - No disulfide bond formation. - Complex purification (soluble form). - Inclusion body, hence protein folding, and denaturation or refolding processing steps are required. - May expose to protease degradation.
Secretory production	- Simple purification. - Improved folding. - N-terminal authenticity. - Soluble protein production. - Prevention from protease degradation.	- Inclusion body may form. - Improper cleavage of signal sequence. - Cell lysis.
Excretory production	- Simple purification. - Improved folding. - N-terminal authenticity. - Soluble protein production. - Prevention from protease degradation.	- Cell lysis. - No excretion usually. - Low protein yield. - Dilution of product.

Source: Choi *et al.* (2006)

Many research groups have focused on the secretory production system to target the recombinant protein production into the periplasmic space based on some exclusive characteristics (Choi & Lee, 2004; Choi *et al.,* 2006). Firstly is that the N-terminal amino acid residue of the secreted product can be identical to that of the naturally secreted gene product, as the signal sequence can be cleaved away by signal peptidases. Second, protease activity is considered to be much lower in the periplasmic space than in the cytoplasm, therefore protein degradation becomes less of an issue. Thirdly, the purification of the recombinant protein could be simplified as the periplasm contains far fewer native host proteins. Finally, correct formation of disulfide bonds can be facilitated because the periplasmic space provides the necessary oxidative environment (Hockney, 1994; Makrides, 1996).

1.2.3 Small scale fermentation using shake flask culture

In process development and optimization in biotechnological industry, shake flasks are small scale reactors of extremely simple mechanical design. They are inexpensive and easy to operate, require only small amounts of material and power, and allow a large number of cultivations to be carried out in parallel (Peter *et al.*, 2006). Due to these attributes, they are primarily used at the early stages of process development, where very decisive experiments are performed. Through the usage of statistically well-designed experiments, shake flask cultures can be harvested and analyzed in large numbers with relative ease, therefore greatly facilitating process optimization (Gerson & Kole, 2001). At this stage, controlled and reproducible experimental conditions are essential in order to ensure that the correct optimized parameters are precisely transferred to the up scaling process of bioreactor fermentation (Freedman, 1970).

1.2.4 Large scale fermentation using bioreactor

A bioreactor may refer to any device or system that supports a biologically active environment. This system is where a chemical process is carried out by organisms, in which biochemically active substances are then derived from such organisms. Bioreactors are widely used for industrial production of microbial, animal and plant metabolites. The process can either be performed under aerobic or anaerobic conditions. These bioreactors are commonly cylindrical, ranging in size from liters to cubic meters, and are often made of glass or stainless steel. They can be further subcategorized into several different types, namely stirred-tank reactor (STR), air-lift fermenter (ALF), dialysis reactor, and chemostat (Takayama & Akita, 1994). In the field of pharmaceutical industry, STR type bioreactor is the preferred conventional mixing vessel frequently used for industrial application.

The components that made up STR (Figure 1.7) basically consist of an impeller, baffles, sparger, and various sensors to detect the conditions of namely pH, temperature, and DO. The impeller functions to stir and mix the culture, causing high turbulence and the formation of a central vortex in the process, in which it is broken down by the baffles that functions to provide a uniform liquid flow. The sparger, which is located directly at the bottom of the impeller shaft functions to break down the inflow aeration into tiny air bubbles that have high surface area over volume ratio in order to provide maximum air transfer rate between the air bubble and the culture. The fitted sensor accessories monitor the culture condition in terms of pH, temperature and DO. The pH condition is controlled by the automated addition of either acid or base to achieve the desired set point. The temperature in the vessel is controlled by removing heat with the means of water circulating through a double-jacketed system. As for the DO percentage, it could be controlled by the

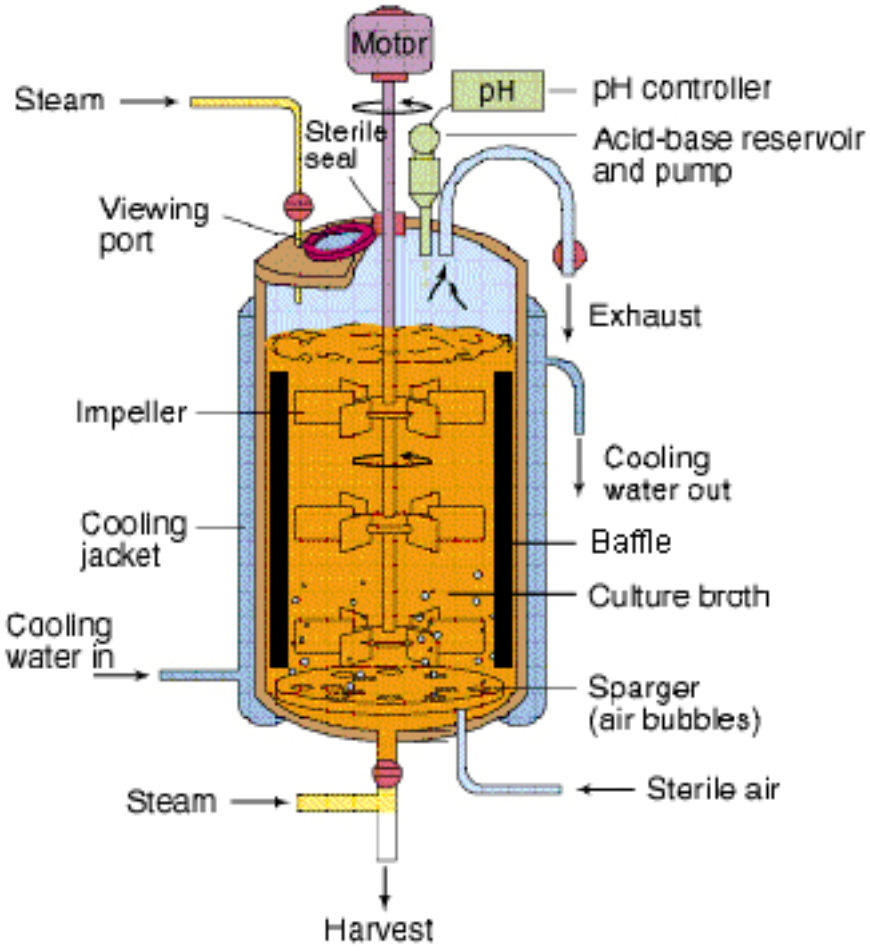

Figure 1.7 Schematic diagram of a stirred-tank reactor (STR)

Source: Microorganisms and Disease Booklet (2010)

application of a cascading system which revs up the stirring speed of the impeller, increasing the aeration or through direct injection of pure oxygen. In addition to all these accessories, a port inlet is usually used by the inoculum to inoculate and start the fermentation process.

The monitoring and controlling of a fermentation running process can be commanded with the assistance of a computer software. In this study, a 5 L bioreactor (Biostat B5, B. Braun Biotech International, Germany) was used to carry out the whole experiment. This bioreactor comes with the Multifermenter Control System Software (MFCS) which functions to collect real-time data points throughout the fermentation process, and also to act as the command center that navigates the bioreactor settings, as well as the controlling of the automated feeding system. In other words, MFCS allows the monitoring and controlling of substrate feeding into the culture. As it name implies, the MFCS is also capable of controlling multiple units of bioreactors at any given time. To hook up the bioreactor with the MFCS, a PC installed with the MFCS-Win software is used as the software interface which allows data exchange between the bioreactor's DCU via a local network cable. Additional sensor accessories are fitted to monitor the culture condition in terms of pH, temperature, DO, and peristaltic pumps for substrate feeding, as well as pH control. Figure 1.8 shows the example of instrument setup for a typical fermentation process.